Drug Delivery

ISSN: 1071-7544 (Print) 1521-0464 (Online) Journal homepage: http://www.tandfonline.com/loi/idrd20

Preparation of folate-modified pullulan acetate nanoparticles for tumor-targeted drug delivery

Hui-zhu Zhang, Xue-min Li, Fu-ping Gao, Ling-rong Liu, Zhi-min Zhou & Qi-qing Zhang

To cite this article: Hui-zhu Zhang, Xue-min Li, Fu-ping Gao, Ling-rong Liu, Zhi-min Zhou & Qi-qing Zhang (2010) Preparation of folate-modified pullulan acetate nanoparticles for tumor-targeted drug delivery, Drug Delivery, 17:1, 48-57, DOI: 10.3109/10717540903508979

To link to this article: https://doi.org/10.3109/10717540903508979

Published online: 16 Dec 2009.

Drug Delivery, 2010; 17(1): 48–57

informa
healthcare

RESEARCH ARTICLE

Preparation of folate-modified pullulan acetate nanoparticles for tumor-targeted drug delivery

Hui-zhu Zhang[1,2], Xue-min Li[1], Fu-ping Gao[1], Ling-rong Liu[1], Zhi-min Zhou[1], and Qi-qing Zhang[1,3]

[1]*Institute of Biomedical Engineering, Chinese Academy of Medical Science, Peking Union Medical College, The Key Laboratory of Biomedical Material of Tianjin, PO Box 25(204), Tianjin 300192, PR China,* [2]*Department of Pharmacology, North China Coal Medical College, 57 Jianshe Road, Tangshan 063000, PR China, and* [3]*Research Center of Biomedical Engineering, Medical College, Xiamen University, 168 Daxue Road, Xiamen 361005, PR China*

Abstract

The purpose of this work was to develop a novel nano-carrier with targeting property to tumor. In this study, pullulan acetate (PA) was synthesized by the acetylation of pullulan to simplify the preparation technique of nanoparticles. Folic acid (FA) was conjugated to PA in order to improve the cancer-targeting activity. The products were characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Epirubicin-loaded nanoparticles were prepared by a solvent diffusion method. The loading efficiencies and EPI content increased with the amount of triethylamine (TEA) increasing in some degree. FPA nanoparticles could incorporate more epirubicin than PA nanoparticles. The folate-modified PA nanoparticles (FPA/EPI NPs) exhibited faster drug release than PA nanoparticles (PA/EPI NPs) in vitro. Confocal image analysis and flow cytometry test revealed that FPA/EPI NPs exhibited a greater extent of cellular uptake than PA/EPI NPs against KB cells over-expressing folate receptors on the surface. FPA/EPI NPs also showed higher cytotoxicity than PA/EPI NPs. The cytotoxic effect of FPA/EPI NPs to KB cells was inhibited by an excess amount of folic acid, suggesting that the binding and/or uptake were mediated by the folate receptor.

Keywords: *Pullulan acetate; folate receptor; epirubicin; nanoparticles; tumor targeting*

Introduction

Chemotherapy in cancer treatment is often limited by the toxicity of the anti-cancer drugs used. This is because both the target cancerous cells and normal cells are non-selectively exposed to the drug, which can lead to unwanted toxic side-effects. Therefore, various drug carriers such as soluble polymers (Janes et al., 2001; Vandamme et al., 2002), polymeric nanoparticles (Chawla & Amiji, 2002; Lemarchand et al., 2004; Yun & Lee, 2005; Gryparis et al., 2007), and liposomes (Park, 2002; Medina et al., 2004) have been investigated in an attempt to increase the therapeutic efficacy and to reduce the toxicity of anti-cancer drugs. Among these systems, the role of natural polysaccharides in developing controlled drug delivery systems has increased significantly and pullulan is gaining lots of attraction towards this application. This is mainly due to its non-toxic, non-immunogenic, and biodegradable properties (Drobe et al., 1997). Pullulan consists of 1–4 and 1–6 glycosidic linkages and is a water-soluble, neutral, linear polysaccharide that cannot self-associate in aqueous solutions due to its solubility in water. So, in any case hydrophobic pullulan derivatives were used as drug delivery carriers (Akiyoshi et al., 1999; Jeong et al., 1999; Na & Bae, 2002; Na et al., 2003a and b). These hydrophobic pullulan derivatives self-associate to form colloidally stable nanoparticles with inner hydrophobic core in water. This hydrophobic core can encapsulate hydrophobic substances like insoluble drugs, DNA, and proteins. The objective of these works was to develop a versatile carrier system for gene and drugs transfer into mammalian cells.

Address for Correspondence: Qi-qing Zhang, Institute of Biomedical Engineering, Chinese Academy of Medical Science, Peking Union Medical College, PO Box 25(204), Tianjin 300192, PR China. Tel/Fax: +86 22 87890868. Email: zhangqiq@xmu.edu.cn

(Received 15 July 2009; revised 08 November 2009; accepted 24 November 2009)

ISSN 1071-7544 print/ISSN 1521-0464 online © 2010 Informa UK Ltd
DOI: 10.3109/10717540903508979

To solve the problem of site-specific targeting for the colloidal systems, some authors have attempted to increase the tissue specificity of colloidal drug carriers by coupling targeting agents. Among the possible targeting agents, folic acid could be exploited to realize delivering drugs into cancer cells. Folic acid (FA) is an essential vitamin whose receptor is over-expressed in various human cancers, including malignancies of the ovary, uterus, breast, kidney, lung, head and neck, brain, and myeloid cancers (Weitman et al., 1992a; Weitman et al., 1992b; Ross & Chaudhuri, 1994; Toffoli et al., 1997; Bueno et al., 2001). Folate receptor (FR) is a 38–40 kDa glycosyl-phosphatidylinositol-anchored glycoprotein with a high affinity for folic acid (Antony, 1996). Tumor-selective targeting carriers have been achieved by combining folic acid with liposomes (Lee & Huang, 1996; Goren et al., 2000; Pan et al., 2003), nanoparticles (Stella et al., 2000; Zhang et al., 2004), and micelles (Yoo & Park, 2004; Liu et al., 2007). The drug delivery system (DDS) endocytosed via a vitamin receptor-mediator inter-action could constitute a more protected pathway for intracellular delivery and could release the drug over a longer period to the endosome, without being rapidly delivered to the lysosomes.

The purpose of the present study was to combine the advantages of hydrophobic pullulan derivatives with folic acid and fabricate a novel tumor targeted nano-carrier. In this study, pullulan acetate (PA) was synthesized to simplify the preparation technique of nanoparticles. Then folic acid was conjugated with PA to improve the cancer targeting activity and internalization of nanoparticles. The nanoparticles were prepared by solvent diffusion method and their physicochemical characteristics were examined by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Epirubicin (EPI) used as a model anti-cancer drug was incorporated into the nanoparticles. EPI release in vitro was studied at phosphate buffered saline (PBS, pH 7.4). Cellular uptake and in vitro cytotoxicity of free EPI and EPI-loaded nanoparticles made from pullulan derivative against KB cells were investigated.

Materials and methods

Materials

Pullulan (Mw = 200,000) was purchased from the Hayashibara Co. (Japan). Folic acid, poly (vinyl alcohol), 4-Dimethylamino-pyridine, and 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Epirubicin·hydrochloride was supplied from Hisun Pharmaceutical Co., Ltd. (Zhejiang, PR China). N, N'-Dicyclohexylcarbodiimide was purchased from Shanghai Medpep Co., Ltd (Shanghai, PR China). RPMI-1640 medium without folic acid was obtained from Gibco (Gibco Co. Uxbridge, UK). KB cell line was purchased from ATCC. All other chemicals were of analytical grade.

Acetylation of pullulan

PA, as hydrophobized pullulan, was synthesized as follows (Jung et al., 2003): 2 g of pullulan, suspended in 20 ml of formamide, was dissolved by vigorous stirring at 54°C. To this solution, pyridine (6 ml) and 7.5 ml of acetic anhydride were then added. The mixture was stirred at 54°C for 48 h. A dark-brown precipitate was thus obtained and purified by re-precipitation with 1000 ml of distilled water and 500 ml of methanol. The solid material was vacuum-dried for 24 h, and a white powder was obtained. The degree of substitution (DS) of PA was determined by ^{1}H NMR method (Naozumi & Mitsuhiro, 2006).

Synthesis of folate modified PA (FPA)

FA was coupled to PA by N, N'-Dicyclohexylcarbodiimide (DCC) and 4-Dimethylamino-pyridine (DMAP) mediated ester formation. The carboxyl groups of FA (1 g) in dried DMSO were activated by adding DCC (700 mg) and DMAP (200 mg); 0.8 g activated FA was added to 20 ml of dried DMSO containing 1 g of PA and reacted for 72 h at room temperature. After 72 h, the reactant mixture was filtered to remove byproduct. This process was repeated three times for 24 h. To completely remove the non-reactants, the supernatant was precipitated in methanol, then centrifuged to separate the precipitated FPA and washed thoroughly with methanol. The precipitated pellet was suspended and dialyzed against 5 mM carborate/bicarbonate buffer (pH 10.0) for 48 h using a dialysis tube (molecular cut-off 12,000), then dialyzed against distilled water for 72 h. The buffer or distilled water was exchanged at 3–6 h intervals. The precipitated FPA was retrieved by filtration and lyophilized to give a yellow powder. The DS of FPA was determined by UV/Vis spectrophotometry in DMSO at 285 nm.

Characterization of the synthesized polymers

The polymers were characterized by proton nuclear magnetic resonance (^{1}H NMR) spectra, which were recorded on a Varian Inova spectrometer (USA) at 500 MHz, and d-DMSO was used as the solvent.

Nanoparticles preparation and drug loading

Nanoparticles were prepared according to a solvent diffusion method (Fessi et al., 1989; Govender et al., 1999; Bilati et al., 2005). The procedure was as follows: PA or

FPA (50 mg) was accurately weighed and dissolved in DMF (5 ml). Epirubicin hydrochloride (EPI·HCl, 5 mg) was dissolved in DMF (1 ml) and added by triethylamine (TEA) to remove hydrochloride. The solution was mixed with PA or FPA solution after stirring in the dark for 12 h, keeping the drug/polymer ratio constant at 1/10 (w/w). The mixed solution was then added to an aqueous solution of 0.5% (w/w) poly (vinyl alcohol) (PVA, 44 ml) by means of a syringe positioned with the needle directly in the medium under moderate magnetic stirring. The freshly formed nanoparticles were filtered (1 μm filters) and then subjected to ultracentrifugation (Avanti J-25, Beckman, USA) at 18,000 rpm for 20 min at 4°C to remove free drug, DMF, and PVA. The pellet was re-dispersed in distilled water. EPI-loaded nanoparticles were dissolved in a mixture of DMSO/H_2O (9/1, w/w). The amount of EPI incorporated into PA or FPA nanoparticles was determined by a UV/Vis spectrophotometer (Spectramax plus384, Micromolecular, USA) at 485 nm.

Transmission electron microscopy (TEM) examinations

The morphologies of PA and FPA nanoparticles were analyzed by TEM (JEM-100CXII, Jeol, Japan). Several drops of the freshly prepared nanoparticles solution containing 0.02 (w/v)% phosphotungstic acid were placed on a copper grid coated with carbon film, and air-dried at room temperature. The observations were carried out with an electron kinetic energy of 80 kV.

Dynamic light scattering measurement

Size and size distribution of the prepared nanoparticles were determined by dynamic light scattering (DLS, BI-90 plus, Brookhaven Ins., NY, USA) using an argon ion laser beam at a wavelength of 488 nm and a scattering angle of 90°. Each measurement was repeated five times. Before measurement, the particle suspension was appropriately diluted.

Drug release rate measurements

The freshly prepared drug loaded nanoparticles were diluted to 1 mg/ml for polymer concentration. The diluted solutions (2 ml) were transferred to dialysis membrane tubes with MWCO of 10,000 Da. The dialysis tube was then immersed in a beaker containing 10 ml of 0.1 M PBS (pH 7.4), which were shaken at a speed of 100 rev/min, and incubated at 37°C. At specific time intervals, the whole medium was removed and replaced by fresh PBS so as to maintain a sink condition. The amount of EPI in the solution was analyzed by UV/Vis spectroscopy at 485 nm.

Cell culture

KB cells, a human nasopharyngeal epidermal carcinoma cell line over-expressing FR (Sadasivan & Rothenberg, 1989), were cultured in T-25 flasks at 37°C in a humidified atmosphere containing 5% CO_2 using folate-free RPMI-1640 supplemented with 5% penicillin–streptomycin and 10% heat-inactivated fetal calf serum. The only source of folic acid in the media was due to the presence of the 10% fetal calf serum.

Confocal image analysis

EPI accumulated in KB cells was localized using a confocal laser scanning microscope (FV1000, Olympus, Japan). The KB cells (2×10^5 cells/ml) were seeded on MatTek culture dishes for 24 h and then treated with free EPI or EPI incorporated nanoparticles. The concentration of EPI was ca. 10 mg/L. After incubation for 2 h, cells were thoroughly washed with PBS solution. Microscopic visualization of live cells was done in new medium. For EPI, maximum excitation was performed by a 488-nm line of internal argon laser, and fluorescence emission was observed above 515 nm with long-pass barrier filter LP-515.

Flow cytometry

KB cells (2×10^6 cells/well) were seeded in 6-well plates. After 24 h, the cells were incubated with free EPI and EPI incorporated nanoparticles at a final EPI concentration of 10 mg/L for 2 h at 37°C. The cells were thoroughly washed with PBS, then released from the plates with trypsin treatment and pelleted (600 g) twice. The cells were examined on a FACScalibur flow cytometer (Becton-Dickinson Ins., NJ, USA). Twenty thousand cells (gated events) were counted for each sample, and EPI fluorescence was detected with logarithmic settings (FL2; Em = 575 nm). Cells were counted as positive if their fluorescence (FL2) was higher than that of 95% of cells from an untreated cell suspension.

Cytotoxicity test

KB cells (5×10^4 cells/ml) harvested from growing cells as a monolayer were seeded onto 96-well plates at 10,000 cells per well, and incubated for 24 h. Free EPI and EPI-loaded nanoparticles made from FPA and PA were dissolved and diluted in folate-free RPMI 1640 growth medium to give final EPI concentrations of 0.01, 0.1, 0.5, 1, 1.25, 2.5, 5, and 10 mg/L. The drug free polymer nanoparticles in RPMI 1640 were diluted to 250, 500, 1000, and 2000 mg/L. The media in the wells were replaced with 100 μl of the pre-prepared samples containing blank nanoparticles or EPI-loaded nanoparticles.

The EPI and EPI-loaded nanoparticles solutions were removed after 2 h of incubation. Upon removal of the drug solutions, the cells were then washed once with 100 µl of PBS solution, and incubated with fresh RPMI 1640 and maintained in 5% CO_2, at 37°C for 46 h. Fresh growth media (90 µl) and 10 µl aliquots of MTT solution (5 mg/ml) were used to replace the mixture in each well after 46 h. The plates were then returned to the incubator and maintained in 5% CO_2, at 37°C for a further 4 h. The growth medium and excess MTT in each well were then removed. DMSO (150 µl) was then added to each well to dissolve the internalized purple formazan crystals. Each sample was tested in six replicates per plate. Three plates were used, making a total of 18 replicates sample. The UV absorbance at 570 nm was measured using a micro-plat reader (Spectramax plus 384, Micromolecular, USA). The results were expressed as a percentage of the absorbance of the non-treated cells.

Folate competition study

KB cells were cultured with the same conditions as that for the cytotoxicity tests, and seeded onto 96-well plates at 10,000 cells density per well, and incubated for 24 h upon seeding. A stock solution containing FPA/EPI nanoparticles was prepared at EPI concentration of 2.5 mg/L. Another stock solution containing 4400 mg/L of free folic acid was also prepared using 2% $NaCO_3$ and filtered with a 0.22 µm pore-size filter. A series of dilutions was then performed on the stock solution containing free folic acid to reach final concentrations of 137.5, 275, 550, 1100, 2200, and 4400 mg/L. The pre-prepared EPI-loaded nanoparticles solution and the diluted free folic acid solutions were subsequently mixed at 9:1 to obtain drug solutions containing 2.5 mg/L of EPI and 13.75, 27.5, 55, 110, 220, and 440 mg/L of free folic acid, respectively. Equal volume of the 2.5 mg/L EPI-loaded nanoparticles solution was also mixed with pure growth medium to obtain drug solution with only 2.5 mg/L EPI in it. The seeded cells were then incubated at 37°C with 100 µl of the drug solutions for 2 h under the presence of 5% CO_2. Following that, the cells were then washed once with PBS solution, and further incubated with fresh growth medium and maintained in 5% CO_2, at 37°C for 46 h. The cell viability after incubation with the drug solution was then assessed using the same MTT assay as above.

Statistical analysis

Statistical analysis was performed by Student's *t*-test for two groups. All results were expressed as the mean ± SD unless noted exceptionally, a probability (p) of less than 0.05 is considered statistically significant.

Results and discussion

Synthesis and characteristics of PA and FPA

The sample of PA, showed by Figure 1, comprising 2.7 acetyl groups per 1 anhydroglucose unit of pullulan, was synthesized to facilitate the preparation of nanoparticles. Then PA was esterified by folic acid to target to FR over-expressed tumor cells. Figure 2 shows typical ^{1}H NMR spectra of PA and FPA in DMSO-d_6. Hydroxyl proton signals, observed at 4.5–5.6 ppm in the ^{1}H NMR spectrum of pullulan (Figure 2a), decreased, and methyl proton signals at 1.8–2.2 ppm, which are assigned to the acetyl groups, appeared in the ^{1}H NMR spectrum of the PA (Figure 2b). Some methine proton signals of pullulan shifted to lower magnetic field by acetylation. These results indicate that acetylation certainly proceeded (Zhang et al., 2009).

The linkage of FA with PA was confirmed by the presence of signals at δ 6.75–8.77 ppm (Figure 2c). 6.75 ppm (*d*, J = 7 Hz, 3′, 5′ H of FA group), 7.63 ppm (*d*, J = 7 Hz, 2′, 6′ H of FA group), 8.77 ppm (*s*, C7-H of FA group) in the ^{1}H NMR spectrum of FPA. Moreover, some methine proton signals of FA shifted to higher magnetic fields due to γ-carboxylic acid of FA was conjugated with hydroxyl group of PA. The content of FA in the conjugates was 6–8 per 100 anhydroglucose units of PA, determined by UV/Vis (DMSO) at 285 nm.

Solubility of pullulan and its derivates was varied. Pullulan was soluble in water and organic solvent, e.g. DMSO, DMF, and pyridine. After acetylation, PA was insoluble in water, but readily soluble in mostly organic solvents except for alcohols. Compared with PA, FPA was insoluble in acetone and THF, which attributed to the higher hydrophilicity and/or polarity of folic acid.

Characterization of PA or FPA nanoparticles and drug loading

PA and its modified materials have been investigated for their possible utility as a drug delivery carrier, due to its favorable biocompatibility and solubility in a variety of organic solvents (Jung et al., 2003; Na et al., 2003a; b; 2004). The nanoparticles were prepared by the diafiltration method in the literature. In the present paper, the solvent diffusion method was used to prepare PA and FPA nanoparticles. This technique is a straightforward technique. Nanoprecipitation occurs by a rapid desolvation of the polymer when the polymer solution is added to the non-solvent. Indeed, as soon as the polymer-containing solvent has diffused into the dispersing medium, the polymer precipitates, involving immediate drug entrapment (Murakami et al., 1999; Bilati et al., 2005).

The morphological structures of PA and FPA nanoparticles were observed using TEM and the size and

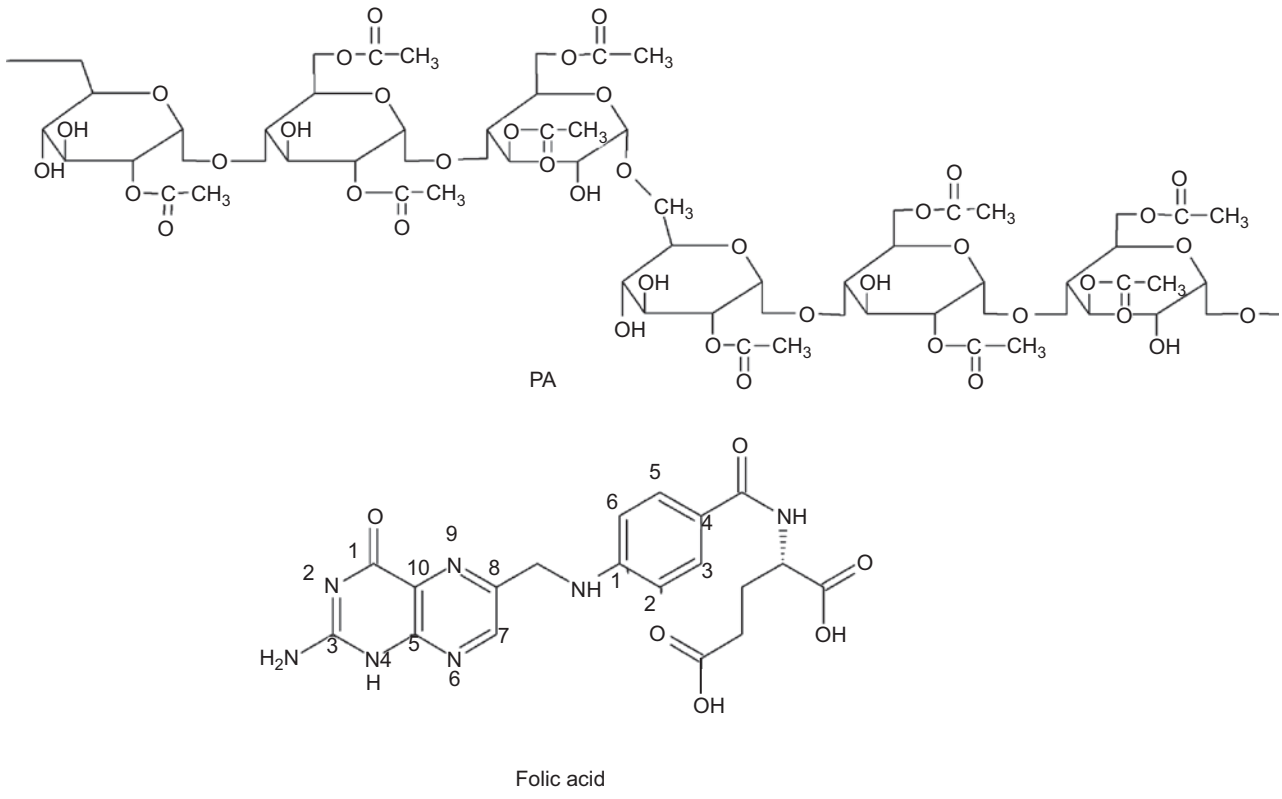

Figure 1. Chemical structures of a repeat unit of PA and folic acid.

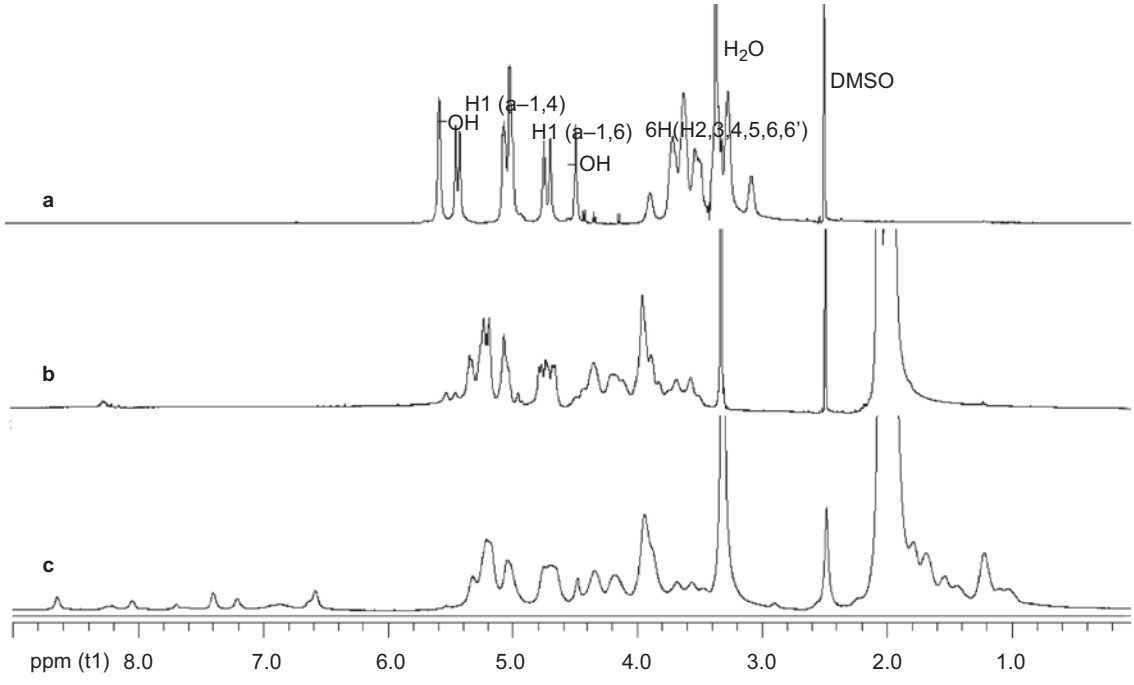

Figure 2. ^{1}H NMR spectra of (a) pullulan, (b) PA, and (c) FPA in DMSO-d_6.

size distributions in distilled water were measured by DLS, as shown in Figure 3. The prepared nanoparticles were found of spherical shape. The mean diameter of PA nanoparticles was 185.7 ± 10.1 nm, and FPA nanoparticles with a folic acid DS of 7.5 was 261.2 ± 33.7 nm. The particle size of FPA nanoparticles was larger than PA due to the increase in the swelling capacity resulting from the hydrophilic property of folic acid. The diameter of

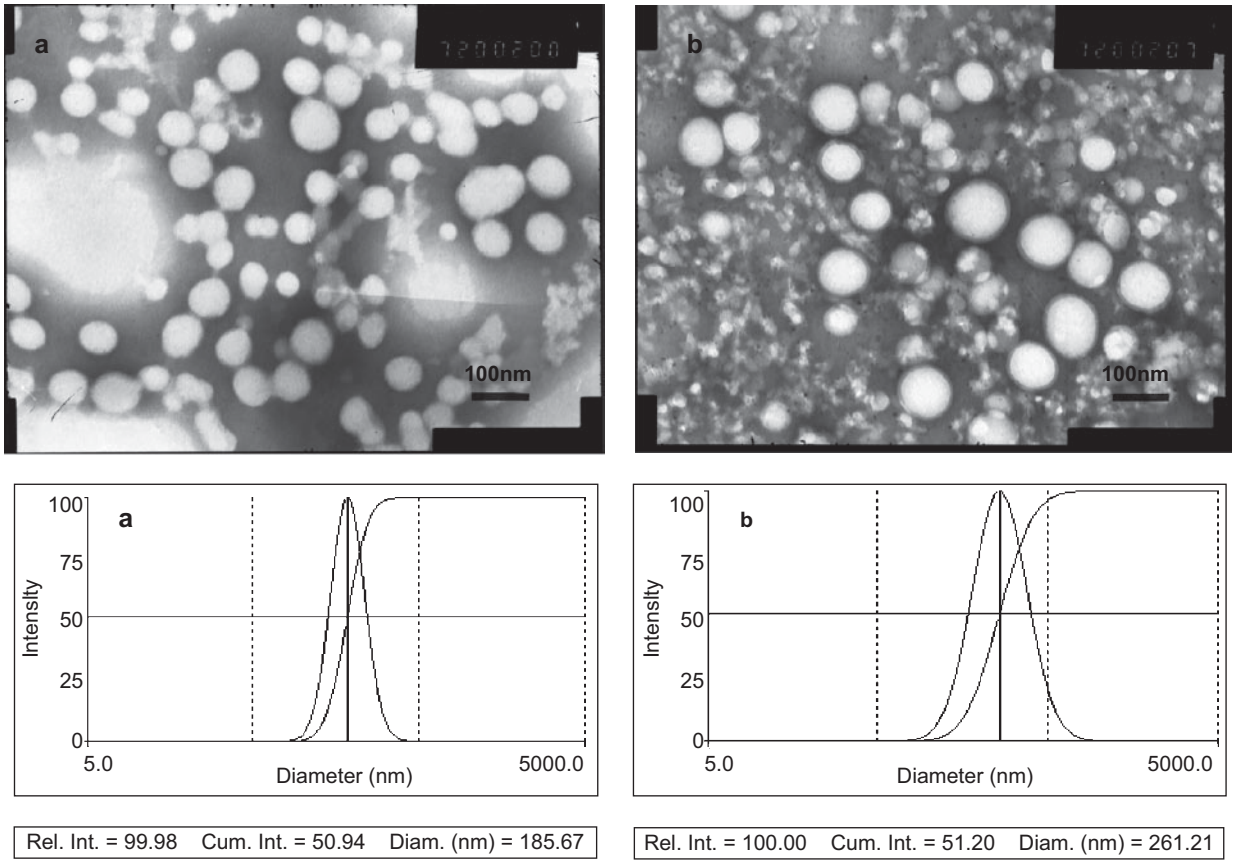

Figure 3. Transmission electron micrograph photographs and size distribution (a) PA and (b) FPA.

nanoparticles observed by TEM was smaller than those obtained by DLS, owing to the diameter obtained by DLS reflected the hydrodynamic diameter of nanoparticles, which swelled in aqueous solution.

The solvent diffusion method is mostly suitable for entrapment compounds having a hydrophobic nature which is soluble in ethanol, acetone, or other water-miscible organic solvents, but displays very limited solubility in water (Bilati et al., 2005). EPI·HCl is hydrophilic due to the protonated form of its amino groups. To obtain a high amount of EPI incorporation into the nanoparticles, non-ionized free amine EPI form may be necessary. TEA of 1~3-times equivalent to the EPI·HCl quantity was added to the organic solvent before preparation of nanoparticles to remove hydrochloride from EPI·HCl. As shown in Table 1, for PA nanoparticles, the incorporated quantity of EPI increased with the level of TEA addition. This was most likely due to the change of drug ionization. When EPI·HCl was encapsulated within PA nanoparticles without using TEA, little EPI (0.01% w/w) was incorporated into nanoparticles, owing to the hydrophilicity of its protonated form. When TEA addition ratio increased, the removal of hydrochloride from EPI·HCl was promoted, therefore, the higher hydrophobicity of EPI was considered to allow physical entrapment in a more efficient manner by hydrophobic interaction (Fukashi et al., 2002). Compared with PA, FPA nanoparticles could entrap more EPI when TEA was added less than 2-times equivalent to the EPI·HCl quantity. The reason might be that the polymer's nature had changed due to the introduction of folic acid. In the following experiments, TEA of 2.0-times equivalent to the EPI·HCl quantity was added in view that the overdosage of TEA would inactivate of EPI.

At the same time, due to the incorporation of drug, the interaction of polymer molecules was decreased. Therefore, the average effective diameter of drug loaded nanoparticles was increased, with drug contents increasing both PA and FPA nanoparticles.

In vitro drug release

Figure 4 showed the in vitro release profiles of EPI from PA and FPA nanoparticles, which exhibited a biphasic release pattern characterized by an initial burst of ~ 36.8 ± 2.2% and 45.4 ± 2.5% of the total entrapped EPI in the nanoparticles at 6 h. It was mainly due to the release of some part of drug, which was absorbed onto the surface of nanoparticles or loosely encapsulated in the hydrophilic domain. The initial burst is followed

Table 1. Particle size and drug-loading contents against various TEA/ EPI molar ratio ($n = 3$).

	PA			FPA		
TEA/EPI (mol/mol)	Drug entrapment (wt%)	Drug content (wt%)	Diameter (nm)	Drug entrapment (wt%)	Drug content (wt%)	Diameter (nm)
0/1	1.0 ± 0.01	0.01 ± 0.001	195.4 ± 5.4	15.92 ± 2.81	1.57 ± 0.28	257.4 ± 5.4
1/1	7.9 ± 1.21	0.78 ± 0.08	209.2 ± 10.3	55.43 ± 1.94	5.25 ± 0.18	357.9 ± 29.5
2/1	54.4 ± 2.93	5.16 ± 0.27	284.3 ± 21.5	71.32 ± 1.81	6.65 ± 0.16	387.5 ± 36.2
3/1	64.8 ± 1.73	6.09 ± 0.20	347.9 ± 38.5	46.52 ± 0.53	4.44 ± 0.04	321.4 ± 13.7

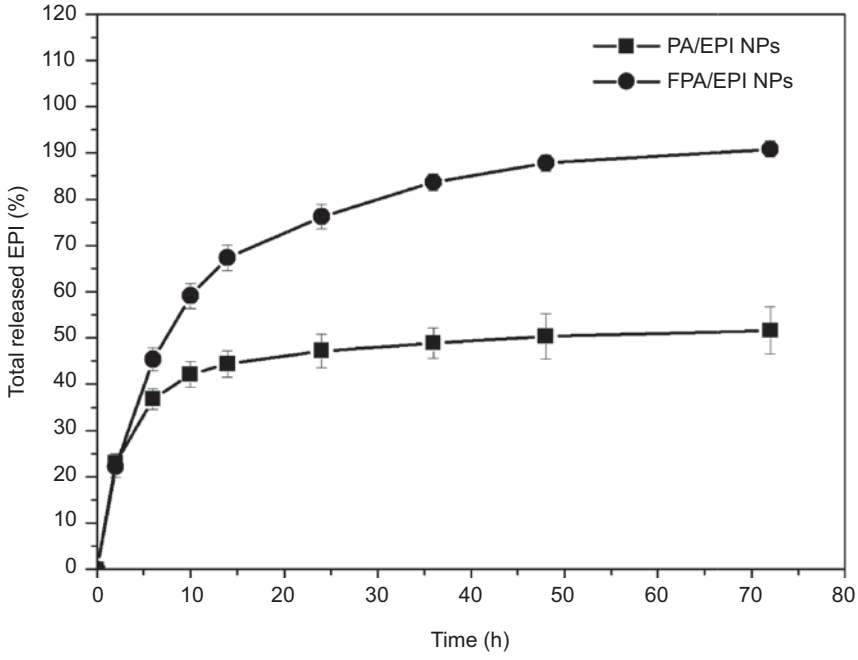

Figure 4. EPI release from PA and FPA nanoparticles at 37°C (PBS, pH 7.4).

by a slow, sustained release. The total amount of drug released from PA and FPA nanoparticles over 72 h were 51.6 ± 5.1% and 90.8 ± 1.8%, respectively. The increased release rate of FPA nanoparticles was attributed to the higher hydrophilicity, owing to introduction of folate. The release medium could easily access the core of the nanoparticle and increase the release rate.

Cellular accumulation studies in KB cells

Confocal microscopy was performed to compare uptake of free EPI, PA/EPI NPs, and FPA/EPI NPs using KB cells in the folate absence incubation medium. As shown in Figure 5, when free EPI was incubated with KB cells for 2 h, epirubicin molecules were transported into the cells through a passive diffusion pathway, and mainly accumulated in the nucleus (Figure 5a). However, fluorescence was observed in the cytoplasm when the cells were incubated with PA/EPI NPs at the same time (Figure 5b). This finding indicates that PA/EPI NPs were internalized by the cells through an endocytosis process, and then escaped from the endosomes and/or the lysosomes to enter the cytoplasm (Panyam & Labhasetwar, 2003; Breunig et al., 2008). In contrast, strong fluorescence was observed in the cytoplasm as well as the nucleus when the cells were incubated with the EPI-loaded FPA nanoparticles (FPA/EPI NPs). The strong signals that appeared in the nucleus were attributed to the EPI molecules released from the nanoparticles. This may be attributed to the fact that drug release from FPA/EPI NPs was much faster than PA/EPI NPs. However, it seems that an equivalent amount of drug is released from PA and FPA nanoparticles within the first 4 h in vitro (Figure 4). The process of drug release from nanoparticles in cells was different from in vitro, which was demonstrated by our previous research (Zhang et al., 2009).

Cellular uptake extents of free EPI and EPI-loaded nanoparticles by KB cells were also evaluated by flow cytometry analysis, as was shown in Figure 6. There was a little difference in cellular uptake of EPI between free EPI and PA/EPI NPs. However, when KB cells were incubated with FPA/EPI NPs, much greater cellular uptake of EPI was observed in the folate-free medium. The flow cytometry results confirmed that FPA/EPI NPs could be targeted to cancer cells over-expressing folate receptors on their surface. Employing folate as a targeting moiety

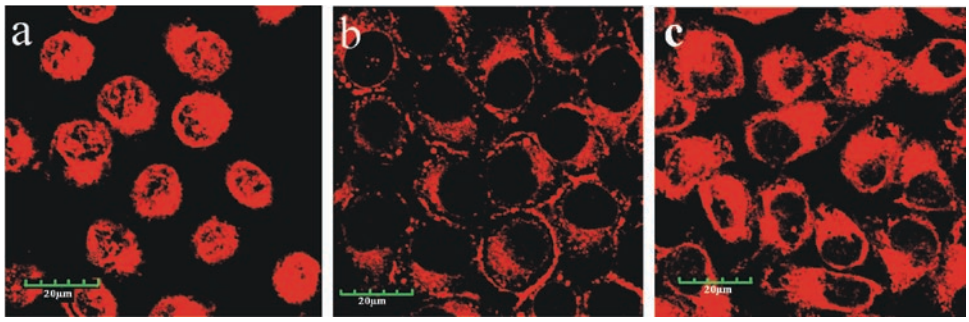

Figure 5. Confocal microscopic images of KB cells incubated with (a) free EPI, (b) PA/EPI NPs, (c) FPA/EPI NPs at an equivalent EPI concentration of 10 mg/L for 2 h at 37°C.

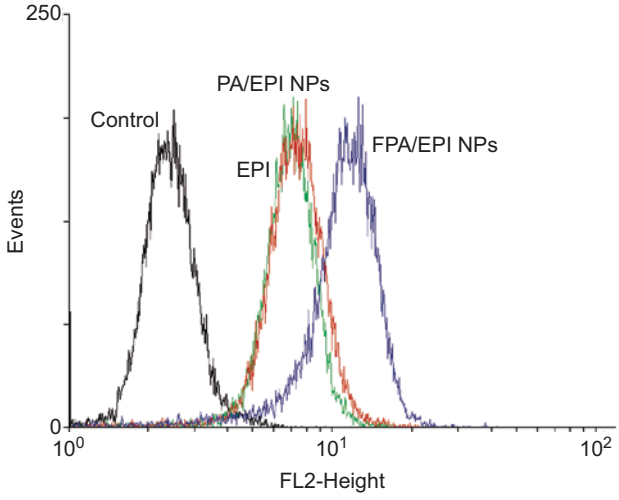

Figure 6. Flow cytometry profiles of fluorescence from cell-associated EPI. KB cells incubated with free EPI (green), PA/EPI NPs (red), FPA/EPI NPs (blue) at an equivalent EPI concentration of 10 mg/L for 2 h at 37°C.

was widely studied in various delivery vehicles, especially liposomes for anti-cancer therapy. In the present study, polymeric nanoparticles with a targeting moiety were utilized as an alternative EPI delivery vehicle, circumventing the instability concern occurring in most cases of liposomes.

Cellular cytotoxicity

KB cells grown in 96-well plates were exposed to serial dilutions of EPI-loaded nanoparticles or free EPI for 2 h, and cell viability was determined by the MTT assay following 46 h further incubation, as described earlier. EPI concentrations leading to 50% cell killing (IC_{50}) were determined from concentration-dependent cell viability curves. According to the data obtained, even after prolonged contact with the cells (4 h), the drug free PA or FPA nanoparticles exhibited low cytotoxicity up to 2 mg/ml.

Cell cytotoxicities of EPI, PA/EPI NPs, and FPA/EPI NPs against KB cells were investigated. EPI concentrations leading to 50% cell-killing (IC_{50}) were determined from concentration-dependent cell viability curves. As shown in Figure 7, FPA/EPI NPs (IC_{50} = 1.12 mg/L) exhibit superior cytotoxic activities to PA/EPI NPs (IC_{50} = 2.75 mg/L). This reveals that folate moieties in FPA nanoparticles played an important role in exerting a cytotoxic effect by binding of FPA/EPI NPs with folate receptors on KB cells and subsequently increasing their intracellular uptake as a result of the receptor-mediated endocytosis. In contrast to EPI-loaded nanoparticles, free EPI showed less cytotoxicity against KB cell line (IC_{50} = 3.92 mg/L), which result from the reduced cellular uptake of EPI, stressing the key role of nanoparticles binding and internalization in enhancement of cytotoxic activity.

Folate competition

In order to further evaluate the role of folate in the cellular uptake of FPA/EPI NPs, KB cells were incubated with the nanoparticles in RPMI 1640 medium containing increasing concentrations of free folate. The cytotoxicity of FPA/EPI NPs against KB cells was inhibited by excess-free folate, and the cell viability increased with increasing folate concentration (Figure 8). For instance, the cell viability of FPA/EPI NPs was ~ 33% at an EPI concentration of 2.5 mg/L, but it was ~ 63% in the presence of 440 mg/L free folate. These findings suggested that free folate molecules prevented the cellular uptake of the nanoparticles by competitive binding to the folate receptors on the cell surface.

Conclusions

Bio-functional polymer folate-modified pullulan acetate was successfully synthesized and utilized to fabricate nanoparticles by a solvent diffusion method for targeted delivery of anti-cancer drugs. The preparation procedure was easy to be operated and practical for large-scale production. The nanoparticles were spherical in nature, and had a narrow size distribution. EPI incorporation behavior was substantially influenced by feed amounts of TEA. Drug release from

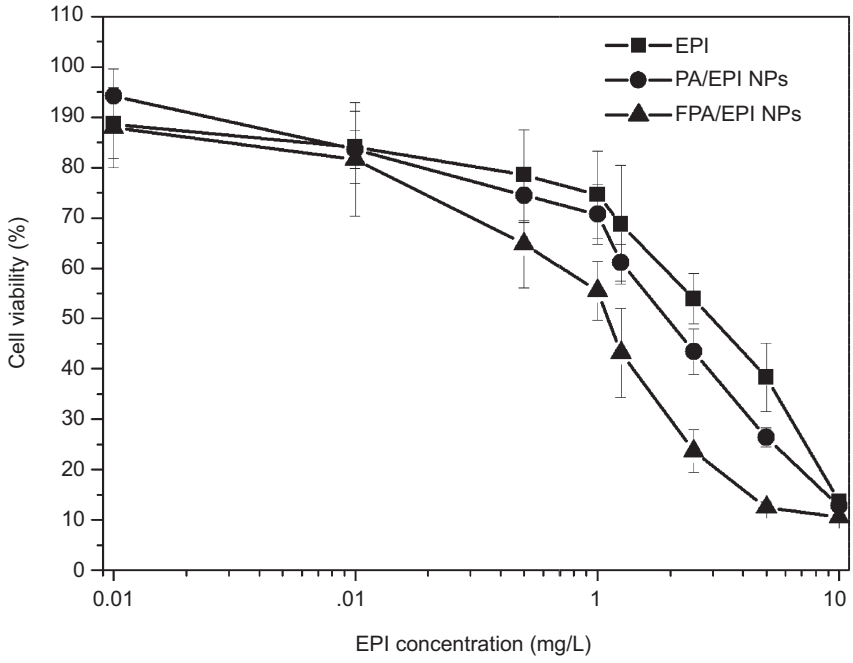

Figure 7. Cytotoxic effects of EPI, PA/EPI NPs, and FPA/EPI NPs against KB cells after 2 h incubation.

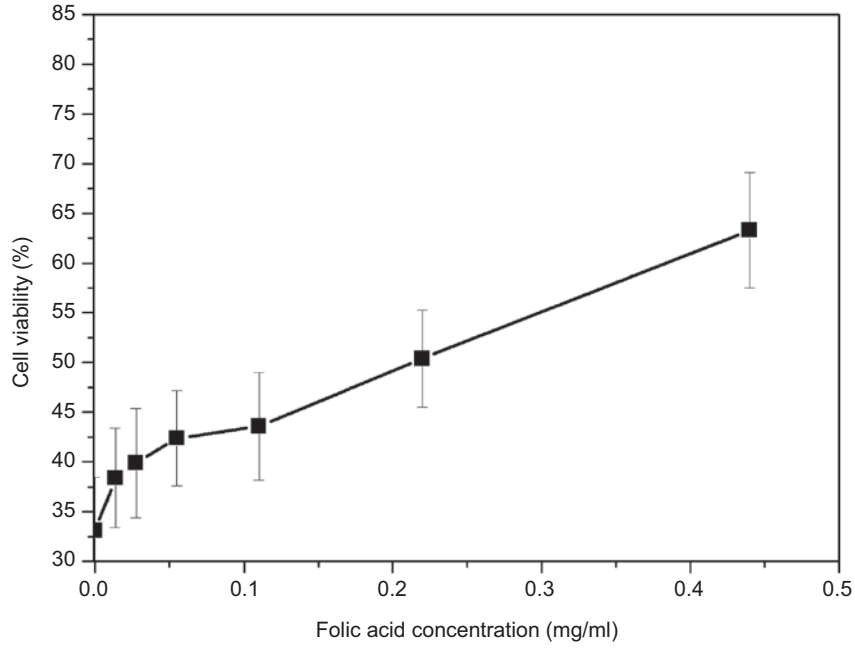

Figure 8. Effect of free folic acid on viability of KB cells incubated with EPI-loaded FPA nanoparticles at EPI concentration of 2.5 mg/L.

FPA NPs was faster than PA. FPA/EPI NPs were taken up by folate receptor-expressing KB cells via the folate receptor-mediated endocytosis process. Greater uptake was observed when compared to PA/EPI NPs, resulting in enhanced cytotoxicity. Folate-conjugated pullulan acetate nanoparticles might be a potential targeted carrier to folate receptor over-expressing cancer cells. Further studies are in progress to test this new drug delivery system in vivo.

Acknowledgements

Declaration of interest

This work was supported by the Major State Basic Research Program of China (No. 2006 CB 933300) and the Doctoral Fund of Ministry of Education of China (No.2006023050). The authors report no conflicts of

interest. The authors alone are responsible for the content and writing of the paper.

References

Akiyoshi, K., Sasaki, Y., Sunmoto, J. (1999). Molecular chaperone like activity of hydrogel nanoparticles of hydrophobized pullulan: thermal stabilization with refolding of carbonic anhydrase B. Bioconjugate Chem. 10:321–4.

Antony, A.C. (1996). Folate receptors. Annu Rev Nutr. 16:501–21.

Bilati, U., Allémann, E., Doelker, E. (2005). Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur J Pharm Sci. 24:67–75.

Breunig, M., Bauer, S., Goepferich, A. (2008). Polymers and nanoparticles: intelligent tools for intracellular targeting? Eur J Pharm Biopharm. 68:112–28.

Bueno, R., Appasani, K., Sugerbaker, D. (2001). The alpha folate receptor is highly activated in malignant pleural mesotherioma. J Thorac Cardiov Surg. 121:225–33.

Chawla, J.S., Amiji, M.M. (2002). Biodegradable poly (o-caprolactone) nanoparticles for tumor targeted delivery of tamoxifen. Int J Pharm. 249:127–38.

Drobe, B., Monot, A., Loeb, M.P., Surysekar K., Kimoto T., Shibuya T., Shiobara S. (1997). Safety studies of a novel starch, pullulan: chronic toxicity in rats and bacterial mutagenicity. Food Chem Toxicol. 35:323–9.

Fessi, H., Puisieux, F., Devissaguet, J.P., Ammoury, N., Benita, S. (1989). Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 55:R1–4.

Fukashi, K., Masayuki, Y., Kiyotaka, S., Teruo, O. (2002). Process design for efficient and controlled drug incorporation into polymeric micelle carrier systems. J Contr Re. 78:155–63.

Goren, D., Horowitz, A.T., Tzemach, D., Tarshish, M., Zalipsky, S., Gabizon, A. (2000). Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. Clin Cancer Res. 6:1949–57.

Govender, T., Stolnik, S., Garnett, M.C., Illum, L., Davis, S.S. (1999). PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Cont Rel. 57:171–85.

Gryparis, E.C., Hatziapostolou, M., Papadimitriou, E., Avgoustakis, K. (2007). Anticancer activity of cisplatin-loaded PLGA-mPEG nanoparticles on LNCaP prostate cancer cells. Eur J Pharm Biopharm. 67:1–8.

Janes, K.A., Calvo, P., Alonso, M.J. (2001). Polysaccharide colloidal particles as delivery systems for macromolecules. Adv Drug Deliv Rev. 47:83–97.

Jeong, Y., Nah, W.J., Na, K., Cho, C.S., Kim, S.H. (1999). Self-assembling nanospheres of hydrophobized pullulans in water. Drug Dev Ind Pharm. 25:917–27.

Jung, S.W., Jeong, Y.I., Kim, S.H. (2003). Characterization of hydrophobized pullulan with various hydrophobicities. Int J Pharm. 254:109–21.

Lee, R.J., Huang, L. (1996). Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J Biol Chem. 271:8481–7.

Lemarchand, C., Gref, R., Couvreur, P. (2004). Polysaccharide-decorated nanoparticles. Eur J Pharm Biopharm. 58:327–41.

Liu, S.Q., Wiradharma, N., Gao, S.J., Tong, Y.W., Yang, Y.Y. (2007). Biofunctional micelles self-assembled from a folate-conjugated block copolymer for targeted intracellular delivery of anticancer drugs. Biomaterials. 28:1423–33.

Medina, O.P., Zhu, Y., Kairemo, K. (2004). Targeted liposomal drug delivery in cancer. Curr Pharm Design. 10:2981–9.

Murakami, H., Kobayashi, M., Takeuchi, H., Kawashima, Y. (1999). Preparation of poly (DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method. Int J Pharm. 187:143–52.

Na, K., Bae, Y.H. (2002). Self-assembled hydrogel nanoparticles responsive to tumor extracellular pH from pullulan derivative/sulphonamide conjugate: characterization, aggregation and adriamycin release in vitro. Pharmacol Res. 19:681–8.

Na, K., Lee, E.S., Bae, Y.H. (2003a). Adriamycin loaded pullulan acetate/sulfonamide conjugate nanoparticles responding to tumor pH: pH-dependent cell interaction, internalization and cytotoxicity in vitro. J Contr Rel. 87:3–13.

Na, K., Lee, K.H., Bae, Y.H. (2004). pH-sensitivity and pH-dependent interior structural change of self-assembled hydrogel nanoparticles of pullulan acetate/oligo-sulfonamide conjugate. J Contr Rel. 97:513–25.

Na, K., Lee, T.B., Park, K.H., Shin, E.K., Lee, Y.B. (2003b). Self-assembled nanoparticles of hydrophobically-modified polysaccharide bearing vitamin H as a targeted anti-cancer drug delivery system. Eur J Pharm Sci. 18:165–73.

Naozumi, T., Mitsuhiro, S. (2006). Synthesis and properties of pullulan acetate, thermal properties, biodegradability, and a semi-clear gel formation in organic solvents. Carbohyd Polym. 63:476–81.

Pan, X.Q., Wang, H., Lee, R.J. (2003). Antitumor activity of folate receptor-targeted liposomal doxorubicin in a KB oral carcinoma murine xenografted model. Pharmacol Res. 20:417–22.

Panyam, J., Labhasetwar, V. (2003). Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv Drug Deliv Rev. 55:329–47.

Park, Y.S. (2002). Tumor-directed targeting of liposomes. Biosci Rep. 22:267–81.

Ross, J.F., Chaudhuri, P.K. (1994). Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Cancer. 73:2432–43.

Sadasivan, E., Rothenberg, S.P. (1989). The complete amino acid sequence of a human folate binding protein from KB cells determined from cDNA. J Biol Chem. 264:5806–11.

Stella, B., Arpicco, S., Peracchia, M.T., Desmaële, D., Hoebeke, J., Renoir, M., D'Angelo, J., Cattel, L., Couvreur, P. (2000). Design of folic acid-conjugated nanoparticles for drug targeting. J Pharm Sci. 89:1452–64.

Toffoli, G., Cernigoi, C., Russo, A. (1997). Overexpression of folate binding protein in ovarian cancers. Int J Cancer. 74:193–8.

Vandamme, T.F., Lenourry, A., Charueau, C., Cheumeil, J.C. (2002). The use of polysaccharides to target drugs to the colon. Carbohyd Polym. 48:219–31.

Weitman, S.D., Lark, R.H., Coney, L.R. (1992a). Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. Cancer Res. 52:3396–401.

Weitman, S.D., Weinberg, A.G., Coney, L.R., Zurawski, V.R., Jennings, D.S., Kamen, B.A. (1992b). Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. Cancer Res. 52:6708–11.

Yoo, H.S., Park, T.G. (2004). Folate-receptor-targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-PEG-folate conjugate. J Contr Rel. 100:247–56.

Yun, M., Lee, Y.L. (2005). Paclitaxel-loaded PLGA nanoparticles: potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. J Contr Rel. 108:244–62.

Zhang, H.Z., Gao, F.P., Liu, L.R., Li, X.M., Zhou, Z.M., Yang, X.D., Zhang, Q.Q. (2009). Pullulan acetate nanoparticles prepared by solvent diffusion method for epirubicin chemotherapy. Colloid Surf B. 71:19–26.

Zhang, L.K., Hou, S.X., Mao, S.J., Wei, D.P., Song, X.R., Lu, Y. (2004). Uptake of folate-conjugated albumin nanoparticles to the SKOV3 cells. Int J Pharm. 287:155–62.